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Ankyrin repeats are found in over 100 proteins, many of which suggest roles for the ankyrin repeat in tumor development. ANK repeats were first identified in a pair of yeast transcription factors (Swi4 and Swi6), and this research is focused upon understanding the role of the ANK repeats within Swi6. We have generated over 30 mutants of the Swi6 ANK domain and analyzed them with respect to their phenotypes, transcriptional activity, and DNA binding. We have found that the ANK core mutants as well as many of the random mutants are temperature sensitive for activity. This suggests that this region of the Swi6 protein is critical for the stability of the protein. We have also noted that many of these mutants affect DNA binding by the Swi4/Swi6 complex. Some cause a dramatic loss of DNA binding activity, but most cause a shift in the mobility of the DNA protein complex which is most easily explained by a global change in the conformation of the complex.

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Linda Breeden 20 Aug 97

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L. Breeden
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The Role of the ankyrin repeats in the Swi4/Swi6 transcription complex of budding yeast.

September 1997

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INTRODUCTION

A large and diverse set of proteins have been identified as containing ankyrin repeats, including several proteins in which the ankyrin repeat motifs may play a role in tumor development. These include Bcl-3, *int*-3, TAN-1, and p16. The *BCL-3* gene encodes a member of the I kappa B family of proteins and rearrangements involving the ankyrin repeat region *BCL-3* have been identified in B-cell chronic lymphocytic leukemias (14). *Int-3*, a mouse proto-oncogene, is a common insertion site for the Mouse Mammary Tumor Virus (MMTV). Activation of the *int-3* ankyrin repeat sequences by MMTV produces poorly differentiated adenocarcinoma of the mammary and salivary glands in mice (10). *TAN-1*, the human *Notch* homologue, was first described as a breakpoint region of t (7,9) gene rearrangements found in T-cell acute lymphoblastic leukemias. This rearrangement places the gene encoding the beta T-cell receptor adjacent to the ankyrin repeat motifs of *TAN-1* (5). p16 is the protein product of the *MTS1* (multiple tumor suppressor-1) gene. It is composed of only a series of four ankyrin repeats and is one member of the cyclin dependent kinase inhibitor family of proteins. p16 can compete with cyclin D and displace the cyclin subunit from its kinase binding site, thereby inhibiting G1 to S progression through the cell cycle. Mutations in p16 have been noted in a variety of human tumors and tumor cell lines, in addition, mutations have been found in the germline of families predisposed to melanoma (9).

Ankyrin repeats are presumed to have a role in protein-protein interaction, though details of these interactions are not available, and the actual function of the motifs is unknown. Ankyrin repeats were originally described by Breeden and Nasmyth (4) in the Swi6 transcription activator of the budding yeast, *Saccharomyces cerevisiae*. Swi6, with its two known protein binding partners, Swi4 and Mbp1, regulates gene expression during the G1 to S transition of the yeast cell cycle. The Swi6/Swi4 heterodimeric complex binds to SCB (Swi4/6 cell cycle box: CACGAAA) elements present in the promoters of *HO* and the G1 cyclins and the Swi6/Mbp1 complex binds to MCB (*MluI* cell cycle box: ACGCGTNA) elements in the promoters of DNA synthesis genes in late G1. Previous evidence suggested that the ankyrin repeat region of Swi6 was necessary for binding of the Swi6/Swi4 complex to DNA and not for the association of Swi6 with Swi4 (17). One hypothesis, therefore, is that the Swi6 ankyrin repeat region recruits additional proteins to the Swi6/Swi4 complex which are required formation of the DNA binding complex.

With this in mind, we focused our initial efforts upon genetic strategies to identify ANK repeat-interacting proteins. These experiments were successful, but they did not identify proteins which directly interact with Swi6. In addition, characterization of the ANK repeat mutants indicate that that this domain palys an important structural role in the Swi6 protein. Aided by an ANK repeat structure that has recently come out, we are taking a more directed approach to understanding the role of this motif in this critical cell cycle regulatory protein.

RESULTS

Our purpose has been to define the function of the ANK repeats. This involved exhaustively mutagenizing the ANK repeats of Swi6 to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and transcriptional activity was determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis. The results of these experiments has led us to the view that most of the ANK repeat domain has a structural role, and to consider alternate strategies for identifying surfaces of the ANK repeat motif which might bind to other proteins.

Our Technical Objectives are to:

1. Generate monoclonal and polyclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats and use them to screen for other ANK repeat-containing proteins.

2. Exhaustively mutagenize the ANK repeats of Swi4 and Swi6, and identify the crucial residues for their activity.

3. Develop a battery of genetic screens to identify mutations that cause loss or deregulation of known Swi4 and Swi6 functions.

4. Perform *in vitro* assays to correlate mutant phenotypes with known biochemical functions.

5. Identify second site suppressors, either within the Swi protein, or within associated proteins.

Writing has been a major focus of work this year, as two manuscripts reporting these studies have been written. One has been submitted and is under revision now. The other will be submitted by the end of the month. These are regarding objectives 2 and 5. In this summary I will focus on our progress on aims 1, 3 and 4, and discuss a natural extension of the aims of this research which we have also engaged in during this year.

TASK 1. Generation of antibodies to and ANK domain.

We have generated new polyclonal and monoclonal antibodies to the Swi6 protein. The polyclonals are very high quality and can be used at a dilution of 1:10,000 on westerns. These will be valuable reagents for isolating and analyzing swi6 mutants levels, though they have not yet been tested in immune precipitation assays. The technical difficulties associated with obtaining large quantities of Swi4 protein have precluded the making of more Swi4 antibodies of any kind. Thus, we have focused our efforts upon understanding the ANK domain function of the Swi6 protein only.

Our monclonal antibodies, and their respective reacitivites with Swi6 on Elisa, Western and immune precipitations (IP) are summarized in Appendix 1 (for methods see (7). There are 23 in all, most of which have been generated with the isolated ANK domain. We have a preliminary result that one of these antibodies 1.2.C6-C11 can be bound to the Swi4/Swi6 DNA complex without disrupting that complex. That had important implications: 1) that the ANK domain was accessible on the surface of Swi6, and 2) that its association with Swi6 did not disrupt the Swi4/Swi6 complex on the DNA. One obstacle to verifying this, and to characterizing all of these monoclonal antibodies in more detail is the fact that the antibody titer is too low in culture supernatants to be useful. We are still attempting to get around this by generating ascities fluid containing these antibodies, but this has not yet been successful.

TASK 3. Analyzing ANK repeat mutants for loss of specific functions *in vivo.*

Our screen for ANK repeat mutants was carried out with an HO:lacZ reporter group, which enabled us to assay Swi6 transcriptional activity with a simple colorimetic assay. We have since assayed these mutants using simplified promoter constructs. Specifically we have asked if these mutants show differential activity upon MCB versus SCB binding sites using specific reporter constructs with differ only in the DNA binding sites. Both sites are known to be bound by Swi6 (1,2,11-13,16), but it is unclear whether their binding would be affected similarly by these mutants. Appendix 2 summarizes the results of those assays.

Most of the ANK mutants show a more extreme loss of MCB- than SCB-driven activation. In fact, the quantitative ONPG assay (15) shows that there is a more than three-fold difference between SCB and MCB transcriptional activity levels for four mutants. These mutations do not localize to a particular region of the ankyrin domain, although each of these mutant has at least two mutations within the fourth repeat. This may indicate a role for the fourth repeat in differentiating between these two complexes.

We have also analyzed the Swi6 ANK mutants for two other phenotypes conferred by swi6 deletion mutants: 1) the inability to recover from alpha factor (3), and 2) hydroxyurea sensitivity (8). Interestingly, although the ANK mutants are highly defective in transcription (see App. 2), they appear to behave as the wild type in these two other assays. This is difficult to understand based upon current knowledge. It is possible that once global searches to identify all the Swi4/Swi6-dependent promoters are identified, candidate promoters that might be responsible for these phenotypes can be deduced and tested.

TASK 4. Biochemical assays of ANK mutant function.

Swi4 binding and DNA binding by the Swi4/Swi6 complex are the only two activities which can be assayed in vitro at the moment. All the mutants that we have obtained have been characterized in regards to these activities. The first step in this process was to determine whether or not the ANK mutants of Swi6 were stable in vivo.

That is, to determine if we could obtain equivalent amounts of each of the mutants from cell extracts so that their in vitro binding activities could be directly compared to wild type. Western analysis (7) was performed, using polyclonal antibodies to Swi6, and we found that most of the mutants were present at levels that were lower than that of the wild type protein (data not shown). This indicates that the mutations in the ANK domain are affecting the susceptability of the protein to intracellular proteases. It also means that direct comparisons of the acitivities of these mutants to wild type from cell extracts cannot be made reliably and quantitatively. Thus, we have resorted to in vitro transcription and translation using a reticulocyte lysate system to produce the proteins. In this cell free system we generate equivalent amounts of each protein for our assays.

Appendix 4 shows the results of band shift assays (17) of DNA binding for wild type and ANK mutants at two different temperatures. All of the ANK mutants being analyzed are temperature sensitive in vivo, and many of them reflect this defect in vitro in the band shift assay. There is a surprising range of effects of these ANK mutants in this assay. Some mutants are extremely defective in DNA binding. Others show a predominant upper complex compared to the wild type cells. Since these assays are carried out with only Swi4 and Swi6 proteins produced from reticulocyte lysates, it is unlikely that the reduced mobility of these upper complexes is due to the addition of another protein in the complex. Rather, we conclude that it is most likely that the ANK mutations are causing a fairly radical change in the conformation of the complex which causes it to migrate or seive its way through the agarose matrix more slowly.

A new approach that we have taken involves a collaboration with structural biologist Dr. Kam Zhang at our institute. Both the temperature instability of the ANK mutants and the evidence we have obtained that they cause a conformational change in the Swi4/Swi6 complex leads us to believe that the ANK domain is largely a structural component of the Swi6 protein. In addition, a crystal struture of another ANK repeat containing protein has recently been published (6). Thus, we have modeled the Swi6 structure using the coordinates generously provided by the authors of that study. This model is of high quality based upon all the available parameters that can be analyzed, and we are now mapping our mutants on this model. Three of the four single mutants we have looked at with this model structure are predicted to be destabilizing and thus validate the model. A fourth mutation appears to be on the surface of the molecule and thus would not be expected to disrupt the structure. This mutant is a good candidate for one which might disrupt a critical interaction with another protein. We are looking for other sights of this type and may embark upon a second round of suppressor analysis to look for proteins which may interact with these surface residues.

CONCLUSIONS

Preliminary results suggest that the ANK domain is accessible on the surface of Swi6, and 2) that its association with Swi6 does not disrupt the Swi4/Swi6 complex on the DNA. This is being verified for publication.

Most of the ANK mutants show a coordinated loss of both SCB and MCB-activation. However, there is a more than three-fold difference between SCB and MCB transcriptional activity levels for four mutants. Each of these mutants has at least two mutations within the fourth repeat. This may indicate a role for the fourth repeat in differentiating between these two complexes.

Although the ANK mutants are highly defective in transcription, they appear to behave as the wild type in two other assays (alpha factor recovery and hydroxyurea sensitivity).

Western analysis indicates that the mutations in the ANK domain are affecting the susceptability of the protein to intracellular proteases.

There is a surprising range of effects of these ANK mutants upon DNA binding. Some mutants are extremely defective in DNA binding. Others show a predominant upper complex compared to the wild type cells. Since these assays are carried out with only Swi4 and Swi6 proteins produced from reticulocyte lysates, it is unlikely that the reduced mobility of these upper complexes is due to the addition of another protein in the complex. Rather, we conclude that it is most likely that the ANK mutations are causing a fairly radical change in the conformation of the complex.

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Appendix 1: Monoclonal antibodies to Swi6

Swi6 ANK repeat domain monoclonals

		Isotype	ELISA	Western	I.P.	Location	
1.2.C6-C11	(Bad 1)	IGG1	√	√		11C(1-8)	11A(31-34)
1.2.D10-B6	(Bad 2)	IGG1	√	√		11C(9-17)	
1.2.E2-A6	(Bad 3)	IGG2a	√			11C(18-26)	
1.4.D1-H3	(Bad 4)	IGG1	√	√		11C(27-32)	
1.4.D9-D6	(Bad 5)	IGG1	√	√	√	11C(33-35)	11A(27-30)
1.1.E5-B5			√	√			
1.4.F12			√	√			
1.4.F8			√	√			
1.6.D1			√	√			
1.6.D8			√	√			
2.1.C7			√	√			
2.1.D3			√	√			
2.1.G3			√	√			
2.1.G4			√	√			
2.3.E10			√	√			
2.3.F2			√	√			
2.3.G6			√	√			
2.4.A5			√	√			
2.4.B9			√	√			
2.4.E9			√	√			

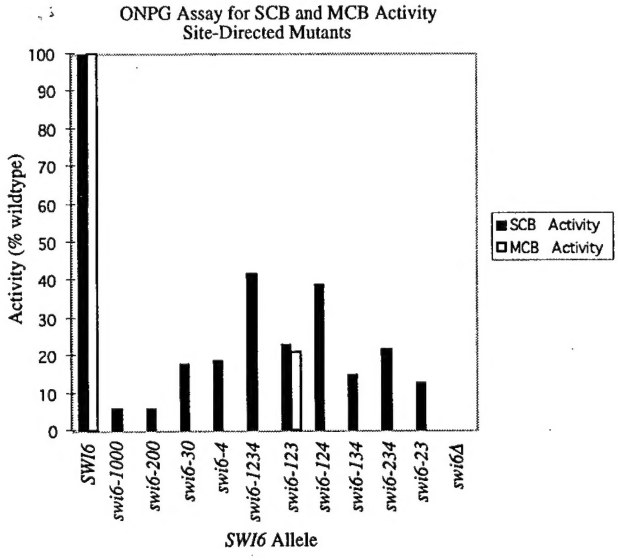
Swi6 (full length) Monoclonal Antibodies

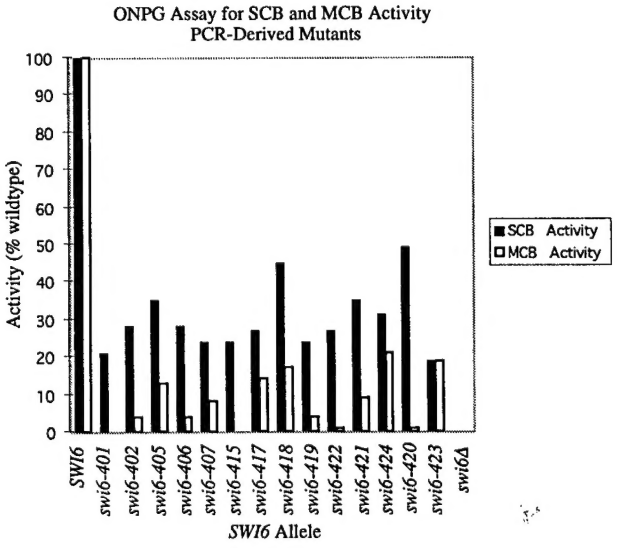
	ELISA	Western	I.P.	Location
4.1.G1	√	√		11A
4.1.A12	√	√		11A
4.4.F12	√	√	√	11A

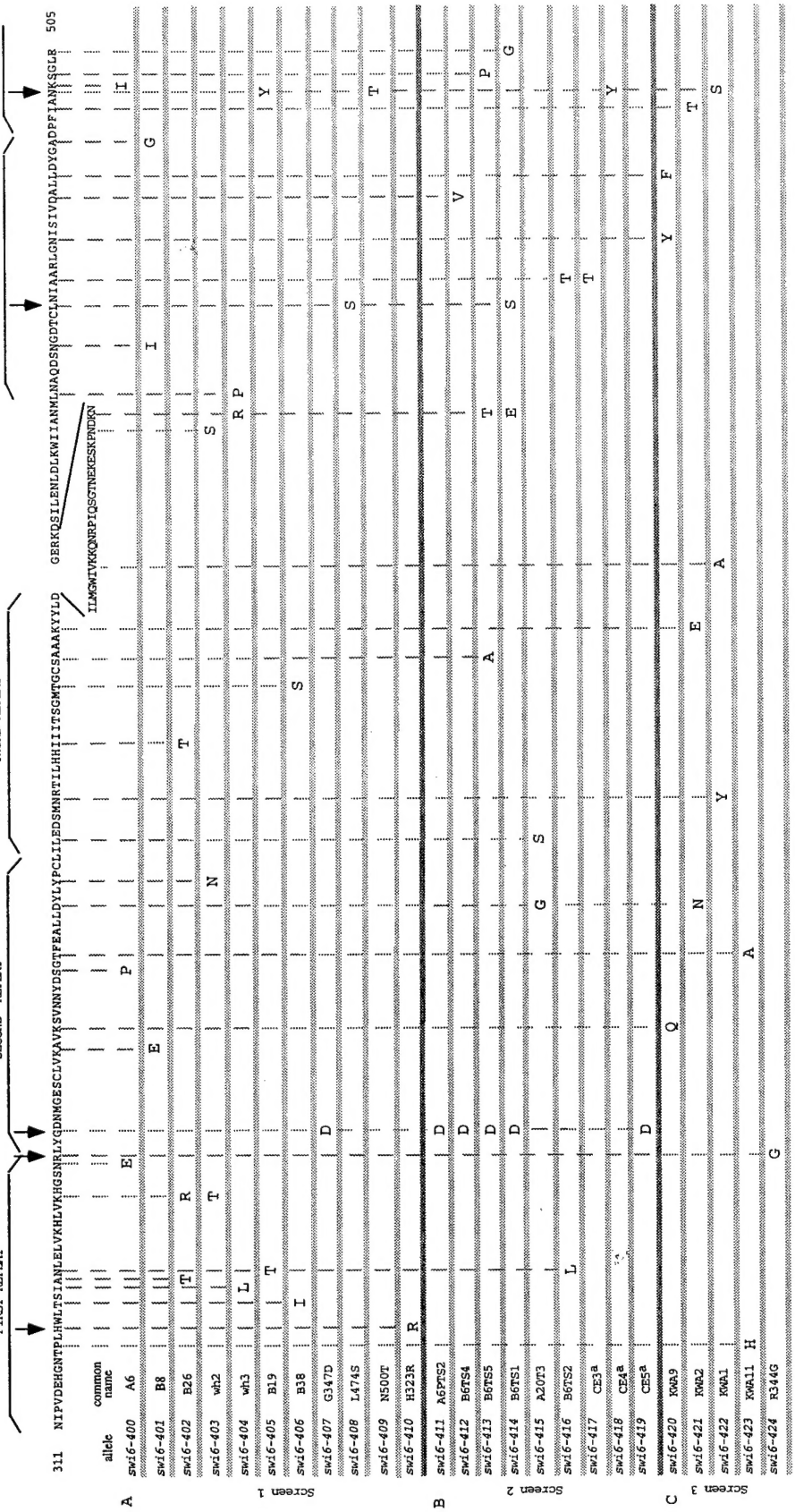
Appendix 2. ONPG assay results for both SCB and MCB reporter constructs.

Using three tandem SCB or MCB DNA binding elements driving the lacZ reporter, we measured the transcriptional activity of selected temperature-sensitive mutants. Activity is reported as a percent of wildtype activity. The wildtype SWI6 gene and each of the mutant alleles was on a low copy plasmid in the presence of the reporter construct, which was expressed from a 2 um plasmid. These mutants are generally more defective in MCB- than in SCB-driven transcription, with the exceptions of swi6-1000 and swi6-200, where both activities are quite low, and swi6-123, swi6-423 and swi6-424, where the levels of activity are higher and fairly comparable. Other mutants, like swi6-1234, show SCB activity at 40% of wildtype, but an undetectable level of MCB activation. The site directed mutations are alanine substitutions in the G_T_L residues in the conserved core of each repeat (1, 2, 3, and/or 4, as indicated in the allele number). The positions of the PCR-generated mutants are shown in the second table in this appendix.

Appendix 2. MCB and SCB driven transcript levels in the Swi6 ankyrin repeat mutants as measured by the ONPG assay.







[a] Mutant was subcloned from a parent plasmid originally isolated from screen 1 or screen 2.

Appendix 3. Bandshift patterns of in vitro translated wildtype and mutant alleles of SWI6.

SWI4 and different alleles of the SWI6 gene are translated in vitro, incubated with labelled SCB-containing DNA and resolved on acrylamide gels as described (17). The upper and lower complexes that are known to contain Swi4 and Swi6 are denoted with arrows. The dramatic shift to higher mobility of some of these mutant complexes is probably due to a conformational change in the complex, rather than the addition of a second protein, because no other yeast proteins are present in this mixture.

Appendix 3. Bandshift pattern variations with *in vitro* translated Swi4 and Swi6 ankyrin repeat mutants. The DNA fragment is a region of the *HO* promoter (-503 to -374) containing four SCB elements.